Mediating Prostate Cancer Metastasis Through the MicroRNA Cluster MiR-23b/-27b

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MEDIATING PROSTATE CANCER METASTASIS THROUGH THE MICRORNA CLUSTER MIR-23B/-27B

By

Meghan A. Rice

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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May 2016
MEDIATING PROSTATE CANCER METASTASIS THROUGH THE MICRORNA CLUSTER MIR-23B/-27B

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Deregulation of microRNAs contributes to progression and metastasis of prostate and other cancers. MiR-23b and miR-27b, encoded in the same miR cluster (miR-23b/-27b), are down-regulated in human metastatic prostate cancer compared to primary tumors and benign tissue. Expression of miR-23b/-27b decreases cell migration, invasion and results in anoikis resistance. Conversely, antagomiR-mediated miR-23b and -27b silencing produces the opposite result in a more indolent prostate cancer cell line. However, neither miR-23b/-27b expression nor inhibition impacts prostate cancer cell proliferation or viability suggesting that miR-23b/-27b selectively suppresses metastasis. To examine the effects of miR-23b/-27b on prostate cancer metastasis in vivo, orthotopic prostate xenografts were established using aggressive prostate cancer cells transduced with miR-23b/-27b or non-targeting control miRNA. While primary tumor formation was similar between miR-23b/-27b-transduced cells and controls, miR-23b/-27b expression in prostate cancer cells decreased seminal vesicle invasion and distant metastases. Gene expression profiling identified the endocytic adaptor, Huntingtin interacting protein 1 related (HIP1R) as being down-regulated
by miR-23b/-27b. Ectopic HIP1R expression in prostate cancer cells inversely phenocopied the effects of miR-23b/-27b overexpression on migration, invasion, and anchorage-independent growth. HIP1R rescued miR-23b/-27b mediated repression of migration in aggressive prostate cancer cells. HIP1R mRNA levels were decreased in seminal vesicle tissue from mice bearing miR-23b/-27b-transduced prostate cancer cell xenografts compared with scrambled controls, suggesting HIP1R is a key functional target of miR-23b/-27b. In addition, depletion of HIP1R led to a more rounded, less mesenchymal-like cell morphology, consistent with decreased metastatic properties. Together, these data demonstrate that the miR-23b/-27b cluster targets hip1r and functions as a metastasis-suppressor in pre-clinical models of prostate cancer.
DEDICATION

To the men who have my heart:

M.A.R.
+
O.A.R.
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<th>Description</th>
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<tbody>
<tr>
<td>ADT</td>
<td>Androgen Deprivation Therapy</td>
</tr>
<tr>
<td>AGO2</td>
<td>Argonaute 2</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens Junctions</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CME</td>
<td>Clathrin-Mediated Endocytosis</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration Resistant Prostate Cancer</td>
</tr>
<tr>
<td>CTRL</td>
<td>Control</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse Large B Cell Lymphoma</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital Rectal Exam</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>EV</td>
<td>Empty Vector</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>HIP1</td>
<td>Huntingtin Interacting Protein-1</td>
</tr>
<tr>
<td>HIP1R</td>
<td>Huntingtin Interacting Protein-1 Related</td>
</tr>
<tr>
<td>HIPK3</td>
<td>Homeodomain-Interacting Protein Kinase 3</td>
</tr>
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<td>JNK</td>
<td>Jun Amino-Terminal Kinase</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low Density Lipoprotein Receptor</td>
</tr>
<tr>
<td>LUC</td>
<td>Luciferase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-Epithelial Transition</td>
</tr>
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<tr>
<td>miR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Cancer</td>
</tr>
<tr>
<td>PAK</td>
<td>P21 Activated Kinase</td>
</tr>
<tr>
<td>PAR CLIP</td>
<td>Photoactivatable Ribonucleoside Enhanced Crosslinking and Immunoprecipitation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>PCA3</td>
<td>Prostate Cancer Antigen 3</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA Induced Silencing Complex</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Luciferase Units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>SCLC</td>
<td>Small Cell Lung Cancer</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small Hairpin Ribonucleic Acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junctions</td>
</tr>
<tr>
<td>TMPRSS-ERG</td>
<td>Transmembrane Protease Serine- ETS Related Gene Fusion</td>
</tr>
<tr>
<td>UTR</td>
<td>UNTRANSLATED REGION</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>VEH</td>
<td>Vehicle</td>
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Chapter I

Introduction

Prostate Cancer

Prostate cancer is the most common cancer affecting men in the United States, with over 220,000 new diagnoses in 2015 alone (Cancer Facts and Figures 2015, cancer.org). Risk of prostate cancer increases significantly with age, and with other risk factors including obesity, familial predisposition and African American heritage. Prostate cancer mortality has dramatically decreased with the discovery of Prostate Specific Antigen (PSA) [1], ensuring most men receive routine screening for this disease, resulting in early detection. Prostate cancer is further diagnosed by digital rectal examinations (DREs), followed by biopsies. Pathology is performed from tumor biopsies to determine tumor grade based upon the Gleason scale. The scale ranges from 1 (normal prostate cells) to 5 (most abnormal). Two grades are given, according to the two most common cell populations, and the scores added for a total Gleason grade of up to 10. Gleason scoring, in combination with PSA and clinical staging of tumor progression, (state of primary tumor, local invasion (seminal vesicles), lymph node invasion, or additional metastasis) combine for a total prostate cancer staging of I-IV. Through these advancements, prostate cancer has become among the easiest to detect cancers, however, the majority of patients are still treated with a watchful waiting approach, as the disease is often slow to progress. Treatments vary widely depending on the staging, but the
armamentarium includes radiation and/or radical prostatectomy as first line therapies, followed by chemical castration via androgen deprivation therapy (ADT), and as a last approach for hormone refractory patients, chemotherapy. Men treated with ADT experience full, albeit temporary recoveries, as most patients relapse within 2-3 years. At this point the disease has progressed to castration resistant prostate cancer (CRPC), for which there is currently no cure.

Additional biomarkers for aggressive prostate cancer, such as PCA3 and TMPRSS2-ERG translocations have begun to show clinical promise, and continue to be investigated [2].

The Metastatic Process

Hanahan and Weinberg have established several hallmarks of cancer, which contribute to aggressive disease. These hallmarks include resisting cell death, sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality and inducing angiogenesis [3, 4].

Metastasis, the process by which tumor cells migrate to and colonize new sites of the body, is responsible for the majority of deaths attributed to most solid tumors, including prostate cancer [5]. Therefore, there is a critical need to understand the metastatic process and identify new therapies targeted to disseminated disease. Metastasis occurs when cells which have acquired an aggressive phenotype allowing for increased self-renewal, invasion, and motility, through oncogenic mutation or genomic instability, escape from their tumor microenvironment [6]. The stages of metastasis can be split between
dissemination and colonization. Disseminating cells intravasate to the bloodstream or lymphatic system through tumor-associated vasculature, allowing them to circulate throughout the body. Cells at this point typically undergo a process referred to as epithelial-to-mesenchymal transition (EMT) allowing them to more easily adapt to colonization of a new site. These cells will extravasate into their newly found site and colonize, developing a satellite metastasis with the help of recruited stromal cells. Whereas EMT is necessary for dissemination, the reverse process, mesenchymal-epithelial transition or MET is frequently observed at later stages of metastasis, allowing for disseminated tumor cells to establish at site of metastasis [7].

Several discrete steps are discernable in the biological cascade of metastasis: loss of cellular adhesion, increased motility and invasiveness, entry and survival in the circulation, exit into new tissue, and eventual colonization of a distant site. [8, 9] While roughly 4 million cells enter the circulation per gram of tumor daily, fewer than 0.01% result in macroscopic lesions [10]. This suggests an overall inefficient process due to the many rate-limiting steps of metastasis. Each step requires careful coordinated expression of genes for a cell to survive.

Metastatic prostate cancer most commonly occurs in bone (pelvis, ribs, spine, mandible and long bones), lungs, liver, brain and lymph nodes- often an early prognostic indicator of tumor invasiveness and metastatic dissemination in prostate and several types of carcinoma [11].
Recent advances in the molecular profiling of cancer using genomic-level approaches have revealed genes whose expression in primary tumors correlates strongly with the likelihood of metastatic recurrence in primary and metastatic carcinomas [12, 13]. These observations have also prompted a reconsideration of how, where, and when cancer cells acquire genes of relevance to metastasis and have raised the possibility that cells with metastatic potential may not be as rare in primary tumors as was originally believed [14].

Cell-cell adhesions are important for normal cellular differentiation and tissue homeostasis. However, disruption of these adhesions in oncogenesis
results in loss of cell signaling and contact inhibition between cells, as well as alterations in cell migration. Compared to normal epithelia, carcinoma cells almost invariably show diminished intercellular adhesiveness [15].

There are three types of adhesion junctions: adherens junctions (AJ), tight junctions (TJ) and desmosomes. The major class of cell adhesion molecules is the cadherins, which are the main components of adherens junctions and desmosomes, and cluster at sites of cell-cell contact in most solid tissues [16, 17]. Classical cadherins are a family of single span transmembrane-domain glycoproteins functioning as cell-cell adhesion molecules. Non-classic cadherins are desmosomal cadherins and protocadherins, usually involved in neuronal plasticity [15].

E-Cadherin is the prototypic cadherin, and is typically lost in progression of epithelial tumors to malignancy. Mechanisms for E-cadherin inactivation in malignant tumors include mutations, epigenetic silencing, increased endocytosis and proteolysis. Although E-cadherin expression can still be found in differentiated tumors in patients, there is an inverse correlation between E-cadherin levels, and tumor grade/patient mortality rates [18, 19]. The frequently observed down-regulation and occasional mutational inactivation of E-cadherin in human carcinomas provided strong support for its role as a key suppressor of this hallmark capability [15, 20]. Loss of E-cadherin is accompanied by increased mesenchymal cadherins, such as N-cadherin in prostate cancer [21], which promotes cell motility and invasion [22]. The intracellular domains of E-cadherin interact with catenin proteins to form a cytoplasmic cell-adhesion complex, which
links to the actin cytoskeleton. Consequences of a loss of E-cadherin are an impairment of cell-cell adhesion, which allows detachment of cells, and nuclear localization of \( \beta \)-catenin, resulting in a gene expression pattern favoring tumor invasion. Mounting evidence indicates multiple reciprocal interactions of E-cadherin and \( \beta \)-catenin with EMT-inducing transcriptional repressors to stabilize an invasive mesenchymal phenotype of epithelial tumor cells [23].

E-Cadherin has several well-known transcription factors: Snail, Slug and Twist, and can signal through a number of pathways including receptor tyrosine kinases [24], Wnt, and Rho GTPases (reviewed in [15]). These transcriptional regulators are expressed in various combinations in a number of malignant tumor types and have been shown in experimental models of carcinoma formation to be causally important for programming invasion and metastasis [23, 25-27]. Included among the cell-biological traits evoked by such transcription factors are loss of adherens junctions, conversion from a polygonal/epithelial to a spindly/fibroblastic morphology, expression of matrix-degrading enzymes, increased motility, and heightened resistance to apoptosis—all traits implicated in the processes of invasion and metastasis. Several of these transcription factors can directly repress E-cadherin gene expression, thereby depriving neoplastic epithelial cells of this key suppressor of motility and invasiveness [28].

Many assays exist for studying the processes of migration and invasion. Wound healing or “scratch assays” were developed to monitor the rate at which cells can move into an artificial void by initiating cell polarization, protrusions, and migration to close the wound. Boyden chamber transwell assays also test cell
migration abilities, using cellular chemotaxis, and attracting the serum starved cancer cells to the lower well, through a porous membrane. This assay has been adapted for invasion as well, adding a Matrigel layer mimicking the basement membrane the cells have to degrade.

**MicroRNA: Biogenesis and Mechanism of Action**

MicroRNAs (miRs) are small noncoding RNAs about 22 nucleotides in length, capable of transcriptional repression or degradation of mRNAs [29]. The discovery of miRs leads back to the identification of a C. elegans gene that affected development, called lin-4, whose corresponding gene product was a small non-protein coding RNA [30]. The importance of this finding was not cemented until the discovery of a second miR, with a human homolog, let-7. To date over 28,000 miRs have been identified (miRbase.org), and have been found to regulate an expanding list of complex biological processes, including cellular proliferation, migration and invasion, and are implicated in all stages of cancer [31-35]. Roughly 40% of human miRs are organized into evolutionarily-conserved clusters, which are coordinately transcribed as discrete polycistronic pri-miRs [36].
miRs are generated through a chain of complex processes beginning with transcription of a precursor, or primary miRNA (pri-miR). This pri-miR is cleaved by an endoribonuclease, Drosha into individual premiRs containing a hairpin
structure. These structures are translocated to the nucleus via Exportin 5, where they are further cleaved of the hairpin by second endoribonuclease, Dicer, into mature miRs. The mature miR is double stranded. Upon unwinding, the mature strand is incorporated into the RNA-induced Silencing Complex (RISC) with Argonaute 2 (AGO2), and participates in inhibition of mRNA translation, usually by binding to the 3'UTR of complementary mRNA sites [29]. Alternatively, if the miR seed sequence has identical complementarity, it may cause mRNA cleavage upon interaction with RISC [37]. Thus, miRs are negative regulators of gene expression, regulating over 30% of mRNAs, and are fundamentally involved in processes such as development, differentiation, cell proliferation, apoptosis, and stress response in species ranging from C. elegans to humans [38]. Alterations in miR levels have been linked to deletions, amplifications or mutations involving the miR loci, attributed to epigenetic silencing or dysregulation of transcription factors targeting specific miRs [38, 39]. Many of these alterations have been correlated with human cancers, as miRs have since been implicated in all of the hallmarks of cancer [40, 41].

Since malignant cells show dependence on the dysregulated expression of microRNA genes, which in turn control or are controlled by multiple protein coding oncogenes or tumor suppressor genes, these small RNAs provide important opportunities for development of future miR based therapies [38]. MiRs additionally can act as independent tumor suppressors or oncogenes [42-44], as evidenced by the initial discovery that miR-15a and miR-16-1 appeared to be initiating events in Chronic Lymphocytic Lymphoma (CLL), and therefore, the first
identified miR tumor suppressors [45]. More recently miRs have been implicated in metastasis suppression or activation, coining a new class of microRNA, metastamiRs. Exampled of these metastamiRs include pro-metastatic miRs- 10b, -373, -520c, -21, -143, and 182 in various cancers [46-52]. Early metastasis suppressing miRs have been identified as well, including miR-335, -206, -146a/b, and -31 [53-56]. These data make it clear that there is both diagnostic and therapeutic potential [57, 58] for miRs in cancer research, and investigation of their role in prostate cancer metastasis is an ongoing area of study [59].

MicroRNAs in Prostate Cancer

Prostate cancer in particular has been linked to the deregulation of a large number of miRs (reviewed in [60]). Lodes et al. identified a panel of fifteen miRs found to be over-expressed in serum from stage 3 and 4 prostate cancer patients compared to normal controls, including miR-16, -92a, -103, -107, -197, -34b, -328, -485-3p, -486-5p, -92b, -574-3p, -636, -640, -766, -885-5p [61]. However, the majority of miRs with altered expression levels appear to be down-regulated in prostate cancer. miR-200 reduces prostate cancer cell growth, and has been linked to the EMT and MET processes in prostate cancer cells [62, 63]. miR-21 expression has been linked to recurrence-free survival in patients following radical prostatectomy [64, 65]. miR-221/-222 are progressively down-regulated in aggressive prostate cancer, and involved in the development and maintenance of the castration-resistant prostate cancer phenotype [66, 67]. Likewise, the reduction of miR-145 expression in prostate cancer was correlated with higher Gleason scores, advanced clinical stage, larger tumor diameter and higher PSA
[68]. Other miRs down-regulated in prostate cancer include miR-1, -133b, -205, -663, -223, -224, -452, -182, -187, -23b and -27b. [69-74]. Many of these are being studied as biomarkers of disease progression, and are inversely coordinated with aggressive disease.

**MiR-23b and MiR-27b**

MiR-23b and miR-27b, two components of the miR-23b-27b-24-1 cluster located on chromosome 9, hereafter termed miR-23b/-27b are down-regulated in several cancer types including esophageal, bladder, lung, renal, colorectal, gastric, and prostate cancers. The most common mechanism for this down-regulation is methylation of the miR-23b-27b-24-1 locus [75-83]. C-MYC is also reportedly involved with transcriptional repression of the miR-23b/-27b promoter, regulating targets mitochondrial glutaminase (GLS) [84] (1.25 fold decrease in our PC3 microarray P=0.02), and apoptotic protease activating factor 1 (Apaf-1), resulting in neuronal apoptosis during hypoxia [85, 86]. Other mechanisms of miR-23b/-27b regulation include E6/E7 silencing [87] and promoter binding by transcription factor AP-1, reducing expression in MDA-MB-231 breast cancer cells and directly regulating several cytoskeletal-remodeling genes including LIMK2, ARHGEF6, CFL2, PIK3R3, PIAU, ANXA2, and TLN2 [88]. While the individual miRs in this cluster are processed individually and have different seed sequences and target mRNAs, regulated gene categories of the three members (miR-23b, -27b and 24-1) are similarly distributed [89].

In prostate cancer specifically, miR-23b/-27b is selectively down-regulated in clinical samples from aggressive human prostate tumors compared to primary
tumors and benign tissue [35, 90-94]. Porkka et al. performed miRNA profiling on prostate cancer cell lines and patient samples to determine a prostate cancer signature, which identified miR-23b and miR-27b among those being down-regulated. Interestingly, miR-27b was only down-regulated in hormone refractory cancer specimens [95]. Fuse et al. additionally confirmed miR-23b, -27b and -24 to be down-regulated in prostate compared to normal clinical samples [77]. Sun et al. determined 90% of metastatic CRPC tumors were characterized by decreased miR-23b/-27b expression, as tested in normal prostate tissues, compared to localized hormone naïve disease and CRPC metastases [96].

Our lab identified miR-23b/-27b as a selective suppressor of key metastatic processes of prostate cancer cells in vitro, including cell invasion, migration and anchorage-independent survival. This mechanism coincided with increased expression of E-Cadherin mRNA and protein, and decreased activity of the Rho GTPase, Rac1. This metastasis suppressing phenotype of miR-23b/-27b in prostate cancer cells is independent of cellular proliferation [97]. The miR-23b locus has been reported as a methylation-silenced tumor suppressor in prostate cancer, suggesting a possible mechanism of action [79, 98],

MiR-23b and miR-27b have independent effects in addition to those of miR-23b/-27b. For instance, miR-23b decreases proliferation of prostate cancer cells, the effects of which are not recapitulated by the cluster [79]. Additionally, there are several conflicting reports of miR-23b/-27b expression in breast cancer-miR-23b/-27b is upregulated and enhances metastasis [99, 100]. The expression
of miR-23b decreased in relation to the increased severity of the lesion and was significantly lower in cervical cancer [101]. miR-23b-3p reversed cancer cell resistance to multiple chemotherapeutics in vitro and sensitize tumors to chemotherapy in vivo [102]. MiR-23b has been implicated in diseases such as diabetic retinopathy, spinal cord injury and multiple cancers, and is down-regulated in auto-immune disease [103]. MiR-27b has been reported to target VEGFC, Notch1, MAPK kinases, c- KIT and SP1 [81, 82, 104]

![Figure 3. The miR-23b/-27b-24-1 cluster](image)

Xu et al, International Journal of Oncology, 2013 [105]

HIP1R and Endocytosis

HIP1R shares 56% identity at the amino acid level to Huntingtin Interacting Protein 1 (HIP1) and is also closely related to the yeast endocytic protein Sla2p [106, 107]. All three proteins participate in endocytosis by coordinating interactions between clathrin, membrane phospholipids and actin [108]. Based on biochemical, genetic and knock out mouse models, HIP1 and HIP1R have partially overlapping functions in endocytosis and other cellular processes [106,
HIP1, which is also overexpressed in prostate cancer, is associated with poor outcome via higher Gleason scores [111, 112], yet *hip1* was not identified as a miR-23b/-27b regulated gene in our microarray analyses. HIP1R is stably associated with clathrin coated pits and vesicles, and the subcellular organization of HIP1R overlaps with clathrin [113, 114]. The COOH-terminal talin-like domain of HIP1R binds F-actin in vitro and is important for the co-localization of the protein with cortical actin in vivo, whereas the association with clathrin-coated pits is mediated by the amino terminal half of the protein [113]. Clathrin light chain directs endocytosis by influencing the binding of the yeast Hip1R homologue, Sla2, to F-actin [115]. HIP1R might therefore represent a physical link between F-actin and clathrin-coated endocytic structures, a function that may be required for spatial organization of endocytosis for an actin-dependent movement of newly formed coated vesicles or both [116].

**Figure 4. HIP1R homologs**  
*Biochemical Society Transactions (2010)* [106]
Endocytosis is an exceedingly complex process, involving recognition of receptors and cell adhesion molecules at the plasma membrane, assembly of clathrin-coated pits, vesicle scission, cargo internalization and intracellular trafficking of endocytic vesicles (in which signaling may proceed) followed by cargo recycling or degradation [117]. Clathrin dependent endocytosis (CDE) is the best characterized endocytic pathway. Certain macromolecules preferentially associate with this pathway and are taken up in clathrin-coated vesicles. Examples are the low-density lipoprotein receptor, transferrin receptor, and epidermal growth factor receptor. [118]

**Statement of Purpose**

MiR-23b/-27b is decreased in metastatic prostate cancer compared to normal and organ-confined prostate cancer cell lines and clinical specimens, making miR-23b/-27b an interesting prospective therapeutic target in prostate cancer. My goals were to determine the in vivo effects of miR-23b/-27b on a preclinical model of prostate cancer, the downstream mediators, and the role this plays in the progression of disease.
CHAPTER II
MATERIALS AND METHODS

Cell Culture
The human prostate cancer cell line ALVA31 was provided by Drs. Stephen Loop and Richard Ostensen at the Department of Veteran Affairs Medical Center, Tacoma, WA [119]. LNCaP cells were obtained from and authenticated by American Type Culture Collection, Manassas, VA. PC3-ML cells were provided by Dr. Alessandro Fatatis at Drexel University College of Medicine [120, 121], and were authenticated by Genetica DNA Labs Inc., Cincinnati, OH, as previously described [122]. All cells were passaged and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (Gibco/Life Technologies, Grand Island, NY), and L-glutamine (Gibco/Life Technologies). PC3-ML cells stably expressing luciferase [122] were cultured in RPMI as described above, in 300 mg/ml G418 (Corning, Corning, NY). shGFP and shHIP1R knockdown cells were derived from ALVA31 or PC3-ML transduced with the corresponding pLKO.1 short hairpin RNA (shRNA) construct and selected over 48 hours using 2.5 mg/ml puromycin. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO2.

Flow Cytometry
GFP expression in transduced ALVA31 and PC3-ML cells was determined by flow cytometry. Untransduced cells were used as a negative control for GFP
expression. Trypsinized cells were permeabilized using 0.05% Trypsin with 0.53 mM EDTA (Cellgro). Flow cytometry was performed on a BD Accuri C6 cytometer using CFlow software according to the manufacturer’s instructions. Analysis was done on 10,000 total gated cells.

**RNA Isolation and Quantitative RT-PCR**

Total RNA was collected using Trizol according to the manufacturer’s protocol (Life Technologies). Five hundred nanograms of total RNA were used for reverse transcription using cDNA Archive Kit as per the manufacturer’s protocol (Applied Biosystems, Foster City, CA). One hundred nanograms of cDNA were used for quantitative PCR of HIP1R and one nanogram for 18S rRNA using the following primers HIP1R (Forward 5’ AGATGCTGTGCGAGGATTGAG 3’; Reverse 5’ TGCAGGCTAGTGGATGTCGTCA 3’) and 18S (Forward 5’ ACCCGTGAACCCCATCGTGTA 3’; Reverse 5’ GCCTCACTAAACCATCCAATCGG 3’). Quantitative real-time PCR was performed using iQ SYBR Green Supermix (Bio-RAD, Hercules, CA) and run on the ABI Prism 7700 (Applied Biosystems by Life Technologies). The comparative threshold cycle method was used to determine relative mRNA expression levels.

For all microRNA level determinations, total RNA was isolated using Trizol as described above. The TaqMan stemloop RT-PCR method using the TaqmanH miRNA reverse transcription kit and the TaqmanH miRNA assays (Applied Biosystems by Life Technologies) was used to assess the expression of miR-23b-3p (Assay Id 000400) and miR-27b-3p (Assay Id 000409). Small nuclear RNA U6 (snU6) served as the internal control for quantification of microRNAs.
**Immunoblotting**

Cellular proteins were extracted and separated on 8% SDS-PAGE gels, and western blot analyses were performed using standard procedures as previously described [123]. Western blotting of β-actin on the same membrane was used as a loading control. The antibodies used were anti-HIP1R (EMD Millipore, Billerica, MS, USA; AB9882), anti-E-Cadherin, anti-Z01, anti-Vimentin, anti-Snail, and anti-β-actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1616.)

**Cell Transfections**

Chemically modified antisense nucleotides (antagomiRs) against miR-23b and miR-27b or control antagomiRs were purchased from Applied Biosystems and transfected at 50 nM using Lipofectamine 2000 (ThermoFisher, Grand Island, NY) according to the manufacturer’s protocol. All luciferase reporter experiments were conducted using the cationic lipid Lipofectamine (ThermoFisher) according to the manufacturer’s instructions. PC3-ML, ALVA31 or LNCaP cells were plated in 35 mm plates 24h before transfection and medium was replaced with unsupplemented DMEM immediately prior to transfection. Then cells were transfected with: 5µg reporter plasmids, pEZXMTO1-HIP1R’3UTR or pEZXMTO1-EV. Also 3ug of CMV-β-galactosidase was transfected to normalize the transfection efficiency. Following 4-6 hours incubation with DNA/lipid complexes, cells were re-fed with RPMI medium supplemented 10% FBS. 48 hours following transfection cells were harvested,
lysed and assessed for luciferase activity using the Promega Dual Glo luciferase assay kit. Beta-galactosidase activity was measured as an internal control for transfection efficiency.

miRNA mimics to miR-23b, -27b and non-coding miR control were reverse transfected using Lipofectamine RNAiMAX transfection reagent according to manufacturers protocol (ThermoFisher.com). In one well of a 24-well plate (perform in triplicate, then pool for experimental plating), 5pmoles miR mimic was diluted in 100µl serum free media with 1µl Lipofectamine RNAiMAX. Following 20-minute incubation, 50,000 ALVA31 cells were added and resuspended. BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Cat. No 14750100) was used to determine transfection efficiency via flow cytometry as described above.

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<th>PC3-ML</th>
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<td>Oncogenic Phenotype (GF independent, Anch. Ind Growth, Migration, Invasion)</td>
<td>Highly Aggressive (Metastatic derivative of PC3)</td>
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<td>Metastatic potential</td>
<td>High (in vivo models)</td>
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<td>Endogenous miR-23b/-27b levels</td>
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Table 1. Cell Models
Plasmids and Lentiviral Production

The human miR-23b/-27b precursor (pMIRNA-23b/-27bGFP) and scrambled control (pMIRNA-Scrambled-GFP) cloned into lentiviral vectors (Systems Biosciences, Mountain View CA, USA) were transfected into LentiX 293T cells (Clontech, Mountain View CA, USA) and viral particles isolated as previously described [97]. GFP expression in transduced ALVA31 and PC3-ML cells was determined by flow cytometry, performed on the BD Accuri C6 cytometer using CFlow software according to the manufacturer’s instructions. pLKO.1 and pLKO.1 shGFP viral vectors were kindly provided by Dr. Priyamvada Rai (University of Miami). The nucleotide sequences used to target HIP1R mRNA were obtained from The RNAi Consortium (TRC) human lentiviral shRNA libraries, available from Sigma-Aldrich Company (Sigma-Aldrich, St Louis, MO, USA.) To generate oligonucleotides for cloning into pLKO.1, the sense and antisense sequences used to target HIP1R were inserted into the following oligonucleotide sequences: Forward 5’-CCGGGCCGTAGATTTGAAAGA ATCTCGAGATTCGTTCAAATCTGACGGGCTTTTTG-3’ and Reverse 5’-AATTCAAAAAGCCCGTACAGATTTGAAAGAATCTCGAGATTCGTTCAAATCTGACGGGC-3’. The oligonucleotides were annealed by heating to 95°C followed by slow cooling to room temperature. The annealed oligos were ligated into Age I- and EcoRI-digested pLKO.1 vector. For selection of stable cell line derivatives, cells were transduced with the appropriate constructs 24 hours after seeding. 48 hours following transduction, cells were selected in 2.5mg/mL puromycin for a
subsequent 48-72 hours. Plasmids pEZXMT01 miRNA 3′-untranslated region (UTR) target expression clones for HIP1R (HmiT021900-MT01) and miRNA Target clone control vector for pEZX-MT01(CmiT000001-MT01) were purchased from GeneCopoeia (Rockville, MD). HIP1R-6myc was a gift from Dr. David Drubin (University of California, Berkeley, CA) [113].

**Orthotopic Xenografts**

Studies involving animals were conducted under a protocol approved by the University of Miami Animal Care and Use Committee. PC3ML-Luc cells, a gift from the lab of Vinata Lokeshwar (Augusta University, Augusta, GA) were transduced with either scrambled or miR-23b/-27b expressing lentivirus as described above (> 95% transduced [97]), and 5x10⁵ cells expressing miR-23b/-27b or scrambled control were injected into the ventral prostates of athymic nude mice (Harlan Laboratories) using a repeating dispenser. Surgical sites were sealed with sutures and 9mm wound clips.

**Ex-Vivo Imaging**

Bioluminescence imaging on the IVIS Spectrum In Vivo Imaging System (Perkin Elmer) began at 7 days post injection (DPI) when wound clips were removed, and continued once weekly until time of sacrifice at day 40. D-Luciferin (Invitrogen) was injected intraperitoneally (150mg/kg) in sterile PBS 5-10 minutes prior to imaging. Following the day 40 imaging time point, mice were euthanized and the prostate and seminal vesicles were excised. Mice (lacking prostates and seminal vesicles) were reimaged to determine distal metastasis signal, read as photon flux (photons/sec/cm²). Prostate and seminal vesicles were placed into
individual wells of a 24 well plate in D-Luciferin to extend the signal life and re-imaged. Bioluminescence imaging emission, read as Photon Flux (photons/second), was quantified using Living Image Software (Perkin Elmer). Tissues were subsequently rinsed with PBS and fixed in 10% buffered formalin for 24 hours. After fixation, samples were dehydrated, embedded in paraffin, sectioned at 4 µm. The sections were stained with hematoxylin and eosin (H&E) and were examined using a Nikon Microphot-FXA microscope and images were captured a Nikon Coolpix 4300 digital camera at 200x Magnification.

**Microarray**

Gene expression profiling was performed using Illumina HumanHT-12 V4 microarrays with PC3-ML cells expressing miR-23b/-27b or scrambled control and LNCaP cells transfected with antagoniR-23b plus antagoniR-27b or control antagoniRs. Three independent replicates for each treatment were used. The preparation of the probes and the microarrays were done according to the manufacturer’s protocol. Results were normalized with quantile normalization and analyzed using Partek Genomic Suite 6.6 software. Only genes with a p-value of ≤0.05 and a fold change of 1.5 times or more were considered for further analysis.

**Cell Proliferation and Viability Assays**

For proliferation, ALVA31, PC3-ML (with stable knockdown of shGFP or shHIP1R) and LNCaP cells (expressing HIP1R or empty vector control) were seeded at an initial density of 10,000 cells/well in a six-well plate. At the indicated time points, the cells were trypsinized and viable cells (those that exclude trypan
blue) were counted using a hemocytometer. Viability assays were performed using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to manufacturers specifications. Cells were seeded at a density of 1,000 cells/well in a 96-well plate, and luminometer readings were taken at 1, 3 and 5 days.

**Migration and Invasion Assays**

Boyden Chamber Migration and Matrigel Invasion Assays (BD Biosciences) were performed using ALVA31 and PC3-ML cells 72 hours after transduction with miR-23b/-27b or scrambled control and LNCaP cells 72 hours after transfection with antagomiR-23b/-27b or control antagomiR, as previously described [97]. Briefly, cells were serum starved for 16 hours then 50,000 cells were seeded into the top chambers of the transwell apparatus with Matrigel coated membranes (24 well insert, 8mm pore size). Medium supplemented with 10% FBS was used as chemoattractant in the lower chambers. After incubation at 37°C for 48 hours, the top chambers were wiped with cotton swabs to remove non-migratory or non-invasive cells. Cells on the lower surface of the membrane were then fixed with cold methanol, stained with 0.01% crystal violet and all cells were counted under a microscope using a light microscope.

**Soft Agar Assays**

The capacity of ALVA31 or PC3-ML cells transduced with shHIP1R or shGFP control to grow in soft agar was evaluated as previously described [97, 124].
PAR-CLIP

Photoactivatable Ribonucleoside Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed by Dr. Omar Flores as previously described [125, 126]. Cells were pulsed with 4-thiouridine (4SU), a non-toxic uridine analog which is incorporated into newly synthesized mRNAs and efficiently cross-linked to mRNA-bound proteins after brief exposure to UV light at 365 nm. The RISC:mRNA complexes were recovered by immunoprecipitation of RISC, via Argonaute and then treated with RNase T1 to remove mRNA sequences which are not bound. The residual mRNA fragments (about 20-40nt) were converted to cDNAs and sequenced using Illumina Hi-Seq. This method was performed on PC3-ML cells overexpressing miR-23b/-27b as well as LNCaP cells with relatively high endogenous levels of miR-23b and miR-27b in order to identify direct (or indirect) targets of miR-23b/-27b in prostate cancer. GenPath pathway analysis software was used to determine enrichment.

Rac1 Activity Assays

Rac1 activity assays were performed on ALVA31 or PC3-ML cells 72 hours after transduction with miR-23b/-27b or scrambled control. GTP-bound Rac1 was separated from GDP-Rac1 using a pull-down approach as previously described [127]. Briefly, cell lysates were incubated with 200mg/ml PBD-GST [p21-binding domain, PBD, of the Rac1/Cdc42 effector PAK (p21-activated kinase)]. GTP-Rac1 was recovered following incubation with glutathione-agarose beads. Complexes were collected, denatured and resolved by SDS-PAGE. Active Rac1 was detected by Western blotting with anti-Rac1 antibodies (Millipore). A total of
5% of the original lysate was also analyzed by immunoblot to determine total levels of Rac1 (GDP-and GTP-bound).

**Immunofluorescence**

For phalloidin staining to assess cell morphology, cells were plated on glass cover slips in 24 well plates and incubated for 5 days at 37°C in 5% CO₂. Plated cells were fixed in 4% paraformaldehyde for 30 minutes, permeabilized for 10 minutes of in 0.2% TritonX-100, and incubated with Alexa Fluor 594 Phalloidin conjugate (Molecular Probes, Life Technologies) in PBS for 30 minutes. Coverslips were mounted using SlowFade Gold antifade reagent containing DAPI (Molecular Probes, Life Technologies).

**3-Dimensional Tumoroid Formation Scaffold Assays**

Cells from a single cell suspension were added to the scaffolds in 96 well plates in a total volume of 100µl, and grown in these conditions for the indicated amount of time. Rounded aggregates of greater than 50mm were counted as tumoroids, for which the diameter was calculated on greater than 20 cells per condition. To compensate for variation in radii, two diameters representing the shortest and longest axes were drawn through the center of each tumoroid and the final diameter was calculated as the average of both values using Image J software [128]. Tumoroid numbers were counted from full planar images taken systematically across each scaffold. Tumoroids and monolayer cells were stained with Whole Cell Stain in Green (Thermo Scientific), scaffolds were placed on glass slides, mounted with coverslips and viewed under a fluorescence microscope.
Imaging and Analysis

Confocal microscopy was done using a Plan Apo N 60x/1.42 Oil objective on the Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan). Confocal image stacks were acquired at 800x800 pixel resolution and a step size of 0.5-2.0 mm. 2D image reconstruction, as well as long vs short axis calculations, and relative cell area was performed using ImageJ (NIH, Bethesda, MD). At least 20 cells per condition were measured for relative cell area. Image merging was performed in Adobe Photoshop CS6. Cell binning was done based on observed cellular phenotypes, and calculated as percent cell population.

Statistical Analysis

All normally distributed data were tested for significance using a Student’s T-test. Data with non-normal distribution were log10 transformed and t-tests were performed. Non-parametric Mann-Whitney tests were done for cell-axis statistical analysis. Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization.
MiR-23b/-27b Decreases Local Invasion and Distant Metastasis of Human Prostate Cancer Cells In Vivo

Previous experiments demonstrated that the ectopic expression of miR-23b/-27b decreases metastatic characteristics in vitro, while silencing miR-23b and miR-27b with antagomiRs has the opposite effect [97]. Therefore, we examined the impact of miR-23b/-27b expression on metastasis of prostate cancer cells in vivo. For these studies, we chose an orthotopic prostate cancer xenograft model, which recapitulates the entire metastatic cascade including loss of cell-cell contact, invasion, intravasation, extravasation, and tumor growth at secondary sites [129, 130]. PC3-ML Luciferase (PC3-ML Luc) cells [122], a metastatic human prostate cancer cell line, were transduced with either lentivirus expressing miR-23b/-27b or a scrambled miR control. I confirmed that miR-23b/-27b inhibited the migration of the luciferase-expressing PC3-ML cells in vitro as had been previously observed for the parental PC3-ML line (Figure 5a)[97]. PC3-ML Luc miR-23b/-27b and scrambled control cells were injected into the ventral prostates of nude mice. Tumor formation was monitored by IVIS imaging. Bioluminescence imaging at the experimental end point (day 40) revealed primary tumor formation in 15/19 mice receiving control cells and 14/19 mice receiving miR-23b/-27b expressing cells. Based on the IVIS imaging, the mice
bearing xenografts of prostate cancer cells transduced with miR-23b/-27b showed a qualitative decrease in metastases compared to controls (Figure 5b). However, metastases could not be quantified effectively given the strong signal coming from the primary tumor contributing to image saturation. Therefore, the prostates were removed from the mice and luminescence measured ex vivo (as seen in Figure 6). Post mortem ex vivo IVIS imaging of prostates showed no significant difference in bioluminescence (Figure 5c), consistent with a lack of effect of miR-23b/-27b on cellular proliferation [97] or primary tumor growth between the two groups (PC3-ML Luc cells transduced with miR-23b/-27b vs. control) (Figure 5b). Additionally, prostate weights and total body weights were not significantly altered between miR-23b/-27b and scrambled expressing tissue (Figure 7). Although no statistically significant effect of miR expression was seen on the growth of the primary tumor, mice bearing miR-23b/-27b transduced PC3-ML Luc cell xenografts displayed significantly less local invasion to the seminal vesicles and decreased distal metastases (Figure 5d,e). At the end of the experiment, the number of metastases per mouse was quantified (as measured by distinct photon regions of interest) and found to be significantly decreased in xenografts from miR-23b/-27b-transduced compared to control cells (Figure 5f). These metastases appeared largely confined to inguinal, lumbar, caudal and splenic lymph nodes as observed during autopsy (data not shown). Histological analysis was performed on prostate and seminal vesicle tissue by H&E staining confirming that tumor invasion into seminal vesicles was largely restricted to the control group (Figure 5g). A closer look at the histology of prostate and seminal...
vesicles tumor tissue revealed a unique immunologic phenotype of the miR-23b/-27b tumors; macrophage recruitment to the tumor perimeter via plethoric blood vessels (Figure 8). Overall, these data demonstrate that miR-23b/-27b decreased local invasion and distal metastasis of prostate cancer cells in vivo.

**MiR-23b/-27b Decreases Expression of HIP1R in Prostate Cancer Cells**

To identify possible targets of miR-23b/-27b, a previous student in the Burnstein lab, Reema Ishteiwy, Ph.D. performed microarray analyses using PC3-ML cells transduced with miR-23b/-27b versus scrambled control, as well as LNCaP, a more indolent prostate cancer cell line with relatively high miR-23b/-27b, transfected with miR-23b and -27b antagomirs versus control antagomiRs. PC3-ML miR-23b/-27b expressing cells exhibited approximately 14 fold greater level of miR-23b compared to cells receiving the scrambled control, as determined by quantitative real-time PCR (qRT-PCR) [131, 132]. LNCaP transfected with antagomiRs to miR-23b and -27b had an almost 60% decrease

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**Figure 5. MiR-23b/-27b decreases local invasion and distal metastasis in an in vivo preclinical model of prostate cancer.**

(a) PC3-ML Luc cells transduced with miR-23b/-27b or scrambled control were tested in Matrigel invasion assays as described in Materials and Methods. (b) PC3ML-Luc cells expressing miR-23b/-27b or scrambled control were injected into the prostate ventral lobes of nude mice. IVIS Imaging was performed at day 40; shown are representative images of mice from both groups (scrambled n=15, miR-23b/-27b n=14). Post-mortem ex-vivo imaging was performed on isolated prostate (c) and seminal vesicles (d). (e) Distal metastasis was defined as all bioluminescence signal remaining post-mortem after removal of prostates and seminal vesicles. (f) Individual metastases were identified as distinct photon regions of interest (ROIs) using Living Image software on IVIS images of mice after removal of prostates and seminal vesicles. (g) Representative images of hematoxylin and eosin immunostaining of formalin fixed tissues. *P<0.05, **P<0.01, ***P<0.001
**Invasion**

- **PC3-ML LUC Scrambled**
- **PC3-ML LUC MiR-23b/27b**

**Ex-Vivo Prostate**

- Log10 (Photons/sec)
- **Scrambled**
- **MiR-23b/27b**

**Ex-Vivo Seminal Vesicle**

- Log10 (Photons/sec)
- **Scrambled**
- **MiR-23b/27b**

**Distal Metastasis**

- Log10 (Photons/sec)
- **Scrambled**
- **MiR-23b/27b**

**Metastases per Nucleus**

- **Scrambled**
- **MiR-23b/27b**

**Vehicle**

**Scrambled**

**MiR-23b/27b**

- **Prostate**
- **Seminal Vesicle**
in miR-23b/-27b. Gene expression changes were analyzed and priority was given to genes that were significantly decreased in PC3-ML miR-23b/-27b transduced cells compared to controls and increased in LNCaP cells transfected with miR-23b and miR-27b antagomiRs vs controls. Also considered was whether candidate target genes contained predicted 3’UTR binding sites for both miR-23b and miR-27b, the existence of multiple putative miR binding sites, and whether the candidate gene is expressed in human prostate cancer.

Figure 6. Ex-Vivo IVIS Imaging.
Following live imaging, animals were sacrificed and autopsied. Organs of interest are washed in PBS, then imaged via IVIS in D-Luciferin. This provides more accurate radiance readings for regions of interest than whole body imaging.
Figure 7. MiR-23b/-27b does not significantly alter whole body or primary tumor weight
(a) Mice were weighed weekly throughout the course of the experiment. Weights were averaged (b) Prostate and seminal vesicle tissue were excised post-mortem and weights were taken. (±SEM)

Using qRT-PCR, several candidates were validated in two independent aggressive prostate cancer cell lines, ALVA31 and PC3-ML, ectopically expressing miR-23b/-27b, as well as in the less aggressive LNCaP transfected with miR-23b and miR-27b antagomiRs. The candidate miR-23b/-27b target, HIP1R was inversely correlated with miR-23b/-27b expression in PC3-ML,
ALVA31 and LNCaP cells [131, 132]. Similarly, HIP1R protein levels were inversely correlated with miR-23b/-27b in prostate cancer cells [131, 132].

**Figure 8. Recruitment of immune response in mice with miR-23b/-27b positive tumors.**
Hematoxylin and Eosin stained tissues imaged at 200x magnification. (a) MiR-23b/-27b prostate indicates immune response surrounding tumor [133], stemming from plethoric blood vessel. (b) Scrambled samples showed evidence of invasion by small cancer cells, as indicated by red arrows, with a visible decrease in epithelial projections. Normal seminal vesicle pseudo-stratified epithelium is pictured for a miR-23b/-27b specimen, shown with surrounding immune response (black arrow), and plethoric blood vessel.

Analysis of publicly available datasets from Oncomine.org (with the help of Phil Miller), revealed that HIP1R mRNA levels are increased in metastatic
compared to organ-confined prostate cancer (Figure 9)[134-136] consistent with our observations that miR-23b/-27b selectively impacts metastasis [97].

Figure 9. Oncomine analyses reveals that HIP1R mRNA is significantly up-regulated in human metastatic prostate cancer compared to primary site disease in multiple datasets.

Prostate cancer mRNA analyses from Oncomine were surveyed for HIP1R in primary prostate cancer as compared to sites of metastasis. HIP1R levels, graphed as log2 median-centered intensity, are listed for three separate significant studies: Grasso Prostate Statistics, Nature 2012/05/20, primary site (59 samples), metastasis (35 samples). T value: 5.537, P-value: 6.65E-7. Chandran Prostate Statistics, BMC Cancer 2007/04/12, primary site (10 samples), metastasis (21 samples). T Value: 5.049, P-value: 1.23E-5. Yu Prostate Statistics, J Clin Oncol 2004/07/15, primary site (64 samples), metastasis (24 samples). T-value: 2.874, P-value: 0.004.
HIP1R is Indirectly Regulated by MiR-23b/-27b

To determine if HIP1R was a direct target of miR-23b/-27b, a luciferase reporter vector containing the wild type 3'UTR of HIP1R was utilized. Overexpression of miR-23b/-27b (as compared to scrambled control) in PC3-ML or ALVA31 cells resulted in similar HIP1R 3'UTR-related luciferase activities as the control luciferase vector lacking the 3'UTR [131, 132]. In addition, the wild type HIP1R 3'UTR luciferase and vector constructs were introduced into LNCaP cells. Consistent with experiments in which miR-23b/-27b was overexpressed, endogenous miR-23b/-27b had no effect on HIP1R 3'UTR-regulated luciferase activity compared to the vector alone [131, 132]. Thus, although miR-23b/-27b regulated HIP1R mRNA and protein levels, the miR cluster did not appear to directly bind and suppress the 3'UTR of HIP1R in prostate cancer cells. These results suggest that miR-23b/-27b indirectly regulate HIP1R expression by potentially targeting an as of yet unidentified gene(s) that acts upstream of HIP1R and regulates HIP1R expression. Interestingly, the level of decrease in HIP1R protein expression greatly exceeded the level of silencing of HIP1R mRNA in the miR-23b/-27b treated cells. This suggests that additional post-transcriptional regulatory mechanisms may be employed that decrease HIP1R protein levels.

New Protein Synthesis is Required for MiR-23b/-27b Mediated Down-Regulation of HIP1R.

To enforce the observation that HIP1R regulation by miR-23b/-27b is an indirect mechanism, I performed a cycloheximide assay. Cycloheximide prevents
new protein synthesis by inhibiting translational elongation. Thus, if an intermediate exists between miR-23b/-27b and HIP1R mRNA regulation, cycloheximide would interfere. ALVA31 cells reverse transfected with miRNA mimics to miR-23b and -27b were treated with 50 µg/ml cycloheximide at 48 hours post transfection for a period of 24 hours. Cells were harvested and qRT-PCR analysis was performed for HIP1R. The no treatment control displayed inhibited HIP1R levels as previously demonstrated [131, 132]. This phenotype was still observed following 24 hours in the vehicle control conditions, as expected. However, following 24 hours in cycloheximide, the decreased expression of HIP1R is attenuated, implying that the regulation of HIP1R is dependent on new protein synthesis (Figure 10).

**Ectopic Expression of HIP1R Increases Invasion and Migration of Poorly Aggressive Human Prostate Cancer Cells**

We previously demonstrated that inhibition of miR-23b/-27b using specific antagomiRs increases migration and invasion of the poorly migratory and less invasive prostate cancer cell line, LNCaP [97]. Using Boyden chamber assays, we found that ectopic expression of HIP1R increased LNCaP cell motility compared to cells transfected with empty vector control ((Figure 11)[131, 132]). In addition, Matrigel invasion assays revealed that ectopic expression of HIP1R increased the invasiveness of LNCaP cells while expression of HIP1R had no effect on proliferation, or cell viability [131, 132](Figure 12). Therefore, overexpression of the miR-23b/-27b target HIP1R in LNCaP cells reproduced the
phenotype observed when miR-23b/-27b levels were reduced in this cell line consistent with a potential role of HIP1R down-regulation by miR-23b/-27b in metastasis suppression.

Figure 10. New protein synthesis is required for miR-23b/-27b mediated downregulation of HIP1R
ALVA31 cells were transfected with miRNA mimics to miR-23b and miR-27b using Lipofectamine RNAiMAX according to the reverse transfection protocol. Cells were seeded the following day in 6 well plates. At 48-hours post transfection, 50µg/ml cycloheximide (CHX) or vehicle (VEH) control were added to media, at which time the 0H timepoint was harvested using Trizol. Cells were harvested 24 hours post treatment, and RNA analysis was performed.
Figure 11. Overexpression of HIP1R in LNCaP cells increases migration

Boyden chamber migration assay was performed on LNCaP cells transfected with pIRES-HIP1R or pIRES-empty vector (EV). Mean cell number (±SD) of a representative experiment, ***P<0.001

HIP1R Depletion Reduces the Metastatic Phenotype of Aggressive Human Prostate Cancer Cells

To evaluate further the importance of HIP1R on prostate cancer invasion and migration, we depleted HIP1R in PC3-ML and ALVA31 cells using an shRNA retroviral construct (shHIP1R) and examined the potential of these cells for invasion and migration. PC3-ML and ALVA31, transduced with an shRNA construct against GFP (shGFP) were used as controls. HIP1R protein levels were greatly decreased in cells stably expressing shHIP1R as compared to shGFP controls as measured by western blot analysis. We observed decreased migration and invasion of both PC3-ML and ALVA31 cells transduced with shHIP1R compared with the shGFP-transduced controls [131, 132] thereby
phenocopying the effects of miR-23b/-27b overexpression in ALVA31 and PC3-ML cells [97]. Consistent with the lack of miR-23b/-27b effects on cell proliferation observed previously [97], HIP1R knockdown did not alter the proliferation, or cell viability of these prostate cancer cell lines [131, 132](Figure 12). Additionally, knockdown of HIP1R resulted in decreased ALVA31 and PC3-ML growth in soft agar (Figure 13), as was observed in these cells expressing miR-23b/-27b [97]. Taken together, this evidence suggests that suppression of prostate cancer invasion and migration by miR-23b/-27b may be mediated through the down-regulation of HIP1R.

**Figure 12. HIP1R does not affect prostate cancer cell viability.**
Cell titer glo assays were performed on LNCaP cells transfected with HIP1R or Empty Vector (EV) control, as well as PC3-ML and ALVA31 cells stably transduced with either a short hairpin (sh) against HIP1R (shHIP1R), or GFP as a control (shGFP). Assays, performed in triplicate, were read at 1, 3, and 5 days following plating in 96 well plates at a concentration of 2,500 cells/well (LNCaP and PC3-ML) or 1250 cells/well (ALVA31). Readings were calculated as a read out of ATP concentration (mg/ml) (±SD).
Figure 13. Depletion of HIP1R decreases anchorage-independent growth in aggressive prostate cancer cells
Anchorage-independent growth was analyzed for PC3-ML and ALVA31 cells depleted of HIP1R using a soft agar assay. Results of three experiments were averaged, and graphed relative to shGFP control (±SEM). *P<0.05

HIP1R Rescues MiR-23b/-27b-Mediated Suppression of Aggressive Prostate Cancer Cell Migration

To determine whether miR-23b/-27b exerts metastasis-suppressing effects through down regulation of HIP1R, we introduced a HIP1R cDNA construct lacking the 3'UTR into PC3ML or ALVA31 cells expressing miR-23b/-27b or scrambled control (Figure 14). This HIP1R construct is not susceptible to miR-23b/-27b mediated depletion. Reema Ishteiwy found that HIP1R expression rescued miR-23b/-27b-mediated suppression of prostate cancer cell migration of both PC3ML and ALVA31 cell lines [131, 132]. Since restoration of HIP1R expression attenuated the metastasis suppressing effects of miR-23b/-27b in prostate cancer cells, modulating HIP1R expression levels appears to be a key mechanism of action of these microRNAs.
Figure 14. Generation of knockdown-resistant HIP1R expression construct. ALVA31 and PC3-ML cells transduced miR-23b/-27b or scrambled control were additionally transfected with a HIP1R cDNA lacking the 3'UTR (pIRES-HIP1R-myc), or a control (pIRES-empty vector (EV)). 48 hours following transfection cell lysates were harvested and subjected to western blot analysis, using actin as a loading control. The lower molecular weight band observed only in cells transfected with pIRES-HIP1R-myc is likely a degradation product.

HIP1R Modifies Cell Morphology in Aggressive Prostate Cancer Lines

Based on HIP1R’s well-recognized role as an endocytic adaptor protein that binds clathrin light chain and coordinates actin reorganization with vesicle dynamics [137], we posited that depletion of HIP1R would affect cellular morphology, specifically involving F-actin. Phalloidin staining (binds to F-actin) was performed to observe possible physiological effects of HIP1R depletion on cell morphology. In PC3-ML and ALVA31 shHIP1R expressing cells compared to the shGFP controls, we observed altered cell shape (Figure 15a and 15b). We quantified these changes by calculating the ratio of the long axis to the short axis of cells stably expressing shHIP1R or shGFP controls and found that HIP1R knockdown led to rounder (less elongated) cells, typically indicative of a more epithelial phenotype. Further, cells area was determined, and cells were sorted...
(binned) based on morphological traits. Interestingly, cell phenotype and area were altered in ALVA31 cells, but not PC3-ML (Figure 16).

Figure 15. Knockdown of HIP1R alters cellular morphology and area of ALVA31, but not PC3-ML cells.
ALVA31 cells transduced with shHIP1R or shGFP were grown for five days in supplemented media, then stained for Phalloidin immunofluorescence, and imaged on a confocal microscope. (a) Perimeter of cells were measured in ImageJ, and used to determine relative cellular area. >20 cells were measured per condition, +/-SD. ***=p<0.001 (b) Cells were binned based on morphological phenotype, and calculated as % cell population.
Figure 16. HIP1R knockdown in aggressive prostate cancer cells results in decreased cell elongation
(a) PC3-ML and (b) ALVA31 cells transduced with shHIP1R or shGFP were grown for five days in supplemented media, then stained for Phalloidin immunofluorescence. Cell shape was measured by determining long axis vs short axis and graphed (±SEM), N=>30 cells per condition. Mann-Whitney test was used to analyze cell axes ratios. **P<0.01, ***P<0.001

Decreased HIP1R mRNA Linked to miR-23b/-27b-Mediated Anti-Invasive Actions In Vivo

To solidify HIP1R as a relevant target for the anti-invasive properties of mir-23b/-27b in vivo, we evaluated expression of HIP1R mRNA in the prostate and seminal vesicles of mice bearing orthotopic xenografts. In the prostate, HIP1R levels, while trending towards decreased mRNA expression, were not
significantly decreased in the samples derived from mice xenografted with miR-23b/-27b-transduced prostate cancer cells (as compared to scrambled controls) (Figure 17). Paired with the discovery that miR-23b and miR-27b are no longer expressed in the prostates of end stage mice (Figure 18), the lack of HIP1R regulation was unsurprising. Conversely, miR-23b and miR-27b levels were higher in the seminal vesicle samples derived from mice xenografted with miR-23b/-27b-transduced prostate cancer cells (as compared to scrambled controls) (Figure 19a). Further, HIP1R mRNA was significantly decreased in the seminal vesicle tissues expressing miR-23b and miR-27b (Figure 19b). These data provide further strong support for miR-23b/-27b-mediated suppression of HIP1R leading to the anti-invasive effects of this miR cluster in vivo.

Figure 17. HIP1R Levels in Primary Prostate Tumors are not significantly decreased.
HIP1R mRNA levels of the prostates from PC3-ML Luc MiR-23b/-27b or Scrambled xenografts implanted into nude mice.
Figure 18. MiR-27b expression lost in end stage prostate tumors
(a) MiR-27b levels of the PC3-ML Luc MiR-23b/-27b transduced cell lines implanted into nude mice. (b) MiR-27b levels of prostate tumors preserved following ex-vivo imaging, compared to vehicle prostate control. Scr n=13, MiR n=11. Tumors were grouped based on IVIS images of the mice into either organ confined disease (with small or large primary tumors, determined qualitatively), or visible metastases.
Figure 19. Locally invaded Seminal Vesicle tissue from MiR-23b/-27b expressing tumors expresses decreased HIP1R.
Seminal vesicle tissue from orthotopic xenograft experiment injecting PC3-ML luciferase cells expressing miR-23b/-27b or scrambled control was homogenized in Trizol and assayed for mRNA. (a) qPCR analysis was performed using Taqman probes to miR-23b and miR-27b. Samples were normalized to SNU6, and controlled to Vehicle tissue. (b) HIP1R human specific Taqman probes were used for qPCR analysis, and normalized to human GAPDH. Mann-Whitney non-parametric test used to determine significance. *=P<0.05, **=P<0.01, ***P=<0.001

Depletion of HIP1R Decreases Rac1 Activity in ALVA31 Cells

Rac1 is a Rho GTPase involved in cell migration, invasion and cell cycle progression. In prostate cancer, Rac1 is hyperactive but not overexpressed in cell lines [127] as well as tumor specimens [138]. MiR-23b/-27b regulates Rac1 activity in ALVA31 and PC3-ML cells [131]. To understand further the role of HIP1R in miR-23b/-27b suppression of prostate cancer migration and invasion, I sought to determine whether HIP1R can affect Rac1 activity in aggressive prostate cancer cells, and found that depletion of HIP1R reduced Rac1 activity of ALVA31 cells without affecting total Rac1 levels (Figure 20). This evidence suggests knockdown of HIP1R mimics the phenotype of ectopic miR-23b/-27b expression in the aggressive prostate cancer human cell line ALVA31. However,
this effect may be specific to ALVA31 cells as knockdown of HIP1R did not alter Rac1 activity in PC3-ML cells (data not shown), suggesting Rac1 may not be a major mechanism involved in HIP1R mediated suppression of invasion and migration.

**Figure 20. Knockdown of HIP1R significantly decreases Rac1 Activity but not total Rac1 levels in ALVA31 cells**

Rac1 activity assays were performed on shGFP ALVA31 or shHIP1R ALVA31 cells. GTP-bound Rac1 was separated from GDP-Rac1 using a pull-down assay as described in Materials and Methods. Complexes containing GTP-Rac1 (active Rac1) were collected, denatured and resolved by SDS-PAGE followed by Western blotting with anti-Rac1 antibodies. Total Rac1 (GDP-and GTP-bound) represents 5% of the original cell lysate. Actin was used as a loading control.
HIP1R Does Not Phenocopy MiR-23b/-27b’s Regulation of E-Cadherin

Our lab previously demonstrated miR-23b/-27b regulation of E-Cadherin protein and mRNA levels [97, 131]. We posited that because HIP1R inversely phenocopies the majority of the miR-23b/-27b phenotype, in conjunction with HIP1R’s link to cell surface protein turnover via endocytosis, that E-Cadherin expression would be regulated by HIP1R. Hypothesizing that knockdown of HIP1R in aggressive prostate cancer cells would increase E-Cadherin protein levels, immunoblotting analysis was performed. Surprisingly, E-Cadherin protein levels remained unchanged, through alterations in cell confluency, and harvest method (Figure 21a). E-Cadherin levels were additionally investigated in LNCaP cells overexpressing and knocking-down HIP1R (Figure 21b and c respectively.) Additionally, E-Cadherin mRNA levels were not regulated by alterations in HIP1R (data not shown).
Figure 21. HIP1R does not phenocopy miR-23b/-27b’s regulation of E-Cadherin protein
Western blot analysis of E-Cadherin, HIP1R, and actin protein levels. Cells were grown to increasing confluencies, and lysates were harvested with RIPA Buffer (a) PC3-ML and ALVA-31 cells were stably transduced with either a short hairpin (sh) against HIP1R (shHIP1R), or GFP as a control (shGFP). (b) LNCaP cells were transiently transfected with HIP1R or an empty vector (EV) control. (c) LNCaP cells were stably transduced with either a short hairpin against HIP1R (shHIP1R), or GFP as a control (shGFP).

HIP1R Does Not Alter Markers of Epithelial-to-Mesenchymal Transition of Prostate Cancer Cell Lines

We thought it interesting that the cell morphology of prostate cancer cells with decreased HIP1R indicated a shift towards an epithelial phenotype, yet E-Cadherin, a canonical marker of the epithelial phenotype, remained unchanged. To further investigate the effects of HIP1R on cellular phenotype, markers of
epithelial-to-mesenchymal transition (EMT) were investigated. Western blot analysis was performed for ZO1- a marker of epithelial phenotype, coupled with Vimentin, a mesenchymal-type marker, in addition to a common E-cadherin transcription factor, Snail. No change in these markers was observed in ALVA31 and PC3-ML knocking down HIP1R, nor in LNCaP cells overexpressing HIP1R for RIPA buffer, used in standard cellular lysis conditions (Figure 22a), or 2X Lamelli Buffer, to limit degradation products of E-Cadherin (Figure 22b). These data contribute to our belief that HIP1R is not regulating migration and invasion through alteration of EMT.

**Figure 22. HIP1R does not affect EMT markers in CaP cell lines.**

Western blot analysis was performed for various markers of the epithelial-to-mesenchymal transition in ALVA31, PC3-ML and LNCaP cells. A. Cells were harvested in RIPA buffer. B. Cells were scraped and harvested directly in 2X Lamelli buffer.
HIP1R Knockdown Affects 3D “Tumoroid” Growth

We have observed a visible alteration in cellular morphology of aggressive prostate cancer cells with decreased HIP1R expression, independently of common mechanisms- E-Cadherin regulation, EMT and activated Rac1 (in PC3-ML cells). It was then our intention of developing a 3-dimensional model to help discern the nuances of this unique phenotype. In collaboration with the lab of Subhra Mohapatra, ALVA31 cells were grown on 3D electrospun fiber scaffolds to form cell tumoroids- cellular aggregates >50μM. Preliminary studies demonstrated a difference in observed phenotype dependent upon the number of cells plated at Day 0, ranging from 4,000 to 8,000 (Figure 23). Proceeding with the low and high confluencies, cells were grown for 6 days to allow thorough differentiation, then stained with whole cell stain and imaged via fluorescent microscopy. ALVA31 cells expressing shHIP1R compared to the shGFP control formed larger tumoroids, again indicative of greater epithelial characteristics (Figure 24 a,b).
Figure 23. 3D Tumoroid formation of ALVA31 cells on 3D electrospun fiber scaffolds.
ALVA31 cells, harvested 5 days post plating on scaffold. Stained with Life Technologies Whole Cell Stain (Green)
Figure 24. HIP1R knockdown affects 3-D tumoroid growth.
(a) ALVA31 cells transduced with shHIP1R or shGFP were grown for 6 days on 3-D, electrospun scaffolds at two cell confluencies, 4000 and 8000 cells. Cells were fixed and stained with Life Technologies Whole Cell Stain (Green). Tumoroids are defined as >50 micrometers (b) Tumoroid diameter was measured in ImageJ by averaging the longest and shortest diameters of each tumoroid (c) Number of tumoroids per condition were calculated.

The Mohapatra lab demonstrated that the growth on their 3p scaffolds induces tumor cells to form tumoroids resembling micrometastatic tumors. The tumoroids undergo EMT by Day 4 in culture as evidenced by increased Vimentin, and loss of E-Cadherin in the very epithelial LLC cell line [128]. We recapitulated this experiment for ALVA31 cells knocking-down HIP1R. However, ALVA31 cells are considerably less epithelial than the LLC cell line used by the Mohapatra lab, and it was more difficult to discern the differences among the cell lines. There
was however, a mild reduction in observed Vimentin by Day 4 in the shHIP1R cell line (Figure 25).

Figure 25. HIP1R knockdown in ALVA31 cells alters EMT in 3D tumoroid formation.
ALVA31 cells transduced with shHIP1R or shGFP were grown for 1 and 4 days on 3-D, electrospun scaffolds at two cell confluencies. Immunofluorescence was performed for E-Cadherin and Vimentin, and scaffolds were costained with DAPI.

Determination of Direct MiR-23b/-27b Targets in PC3-ML Cells

PAR-CLIP was performed by Dr. Omar Flores on PC3-ML cells transduced with miR-23b/-27b compared to the relatively indolent LNCaP cells with high endogenous miR-23b/-27b. I then performed cross-analysis of the miR-23b and miR-27b predicted targets to determine those that were common amongst the two cell lines (Figure 26). 25 genes that are predicted targets of miR-23b or miR-27b were found to overlap in both cell lines- 2 targets of miR-23b, and 23 targets of miR-27b (Table 2). These genes were compared against our microarray analysis, however, and no common targets were observed,
suggesting the mRNA of the predicted direct targets are not regulated by miR-23b/-27b.

The miR-23b/-27b predicted direct targets were then analyzed in pathway analysis software (GenPath UM) to determine any enrichment by miR-23b/-27b for common signaling profiles or pathways. The gene list was significantly enriched for involvement in Glycerophospholipid metabolism (p=0.00044), Actin cytoskeleton (0.0026), and to a lesser degree, sphingolipid, glycerolipid and purine metabolism (P<0.05), suggesting future exploration of these pathways to study the mechanism of miR-23b/-27b actions.

Figure 26. Exploring direct targets of miR-23b/-27b via PAR-CLIP
PAR-CLIP analysis was performed on LNCaP cells compared to PC3-ML stably transduced with miR-23b/-27b. Predicted targets of miR-23, miR-27 and miR-24 were isolated from RNA seq in both cell lines, and then the hits were compared between both cell lines. Overlapping genes predicted to target a member of the miR-23b/-27b-24-1 cluster were isolated.
Table 2. PAR-CLIP hits
The 25 overlapping genes that are predicted targets of miR-23b/-27b.

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 CHAPTER IV  
DISCUSSION

MiR-23b/-27b Inhibits Metastasis of Prostate Cancer In Vivo

miR-23b/-27b has been characterized as a metastasis suppressing miR cluster in prostate cancer [97]. Interestingly miR-23b/-27b does not confer a less aggressive phenotype through any effects on cellular proliferation or viability. It is a conceptual inconsistency that proliferative growth advantage is not required for drivers of metastasis [14]. Meaning, cells that have lost miR-23b/-27b expression within the primary tumor mass do not proliferate more effectively within the primary tumor, and therefore do not increase their representation. Rare cells in primary tumors which happen to acquire metastatic abilities would therefore remain rare- and yet given the low success rate of metastasis enough cells escape to make miR-23b/-27b loss an oncogenic event.

I explored the potential for the miR-23b/-27b cluster to serve as a metastasis suppressor in vivo to complement previous studies from our laboratory demonstrating that miR-23b/-27b decreases migration, invasion and anoikis resistance in multiple prostate cancer cell lines [97]. I also sought to understand the mechanism responsible for the anti-metastatic action of this miR cluster in prostate cancer, as similar metastasis suppressing miRs have been described for aggressive breast cancer [139, 140]. Expression of miR-23b/-27b in the metastatic human prostate cancer cell line PC3-ML was sufficient to
decrease seminal vesicle invasion and distal metastasis in an orthotopic xenograft model of prostate cancer. This preclinical model was chosen as PC3-ML cells reproduce the full metastatic cascade following implantation into the prostates (orthotopic xenografts) of immunocompromised mice. Orthotopic xenografting of these cells results in reliable local invasion to seminal vesicles and lymph node metastasis, as is common in aggressive cases of human prostate cancer [141]. Thus, the orthotopic xenograft model faithfully reproduces these aspects of the disease in humans. A limitation of this model is that PC3-ML cells do not typically metastasize to bone as will prostate cancer cells following intracardiac or intratibial injection. Yet, such current preclinical models of prostate cancer bone metastasis circumvent early steps in the metastatic process. That miR-23b/-27b decreased prostate cancer seminal vesicle invasion and distal metastases, coupled with our previous in vitro data [97], provides strong support for the contention that the miR-23b/-27b cluster functions as a metastasis suppressor.

Additional findings from these in vivo studies suggest miR-23b/-27b may be playing a role in immune cell recruitment. Tumor pathology from the H&E stained miR-23b/-27b prostates and seminal vesicles was analyzed by Teresita Reiner, and compared to the respective scrambled controls. It was observed that the prostates and seminal vesicles from mice bearing miR-23b/-27b transduced xenografts illicited an immunological response, as these mice displayed evidence of plethoric blood vessels around the tumor periphery, allowing for the recruitment of macrophages. While tumor associated macrophages are often
linked to aggressiveness in cancer, macrophages in rare cases can instead mount an immune response against the disease [142]. miRNA have been recently implicated in modulation of many aspects of the tumor microenvironment, including tumor-related inflammation and immune cells (reviewed in [143]). miR-23a and -23b were shown to regulate IL6R in prostate cancer and gastric adenocarcinoma cell lines [94, 144], and IL6 is sufficient to promote tumor associated macrophage (TAM) generation [145].

This phenotype requires further investigation. Immunohistochemical analysis of immune factors can be tested to determine the amount of immunological activation. Additionally, in vitro conditioned media experiments would be helpful to determine if miR-23b/-27b expressing cells are releasing immune recruiting factors, or miR-23b and miR-27b themselves to the surrounding tumor microenvironment.

**Downstream of MiR-23b/-27b: Discovery of Functional Target HIP1R and Potential Mechanisms of Action**

To understand the mechanisms of miR-23b/-27b-mediated metastasis suppression in prostate cancer, we sought to identify miR-repressed target genes that exert pro-metastatic activities. Microarray analyses of prostate cancer cell lines either expressing miR-23b/-27b (PC3-ML) or transfected with miR-23b and -27b antagomiRs (LNCaP), followed up with qRT-PCR validation, identified HIP1R as a novel target of miR-23b/-27b in prostate cancer cells [131]. HIP1R is best recognized as an endocytic adaptor with roles in clathrin-mediated endocytosis and vesicle trafficking [146]. Data mining from multiple microarray
studies of human prostate cancer samples indicates that HIP1R is overexpressed in metastatic tumors compared to organ-confined prostate cancer tumors (Figure 3).

Depletion of HIP1R in aggressive prostate cancer cell lines recapitulated the anti-metastatic effects of miR-23b/-27b, including decreased migration, invasion, and anchorage-independent growth [131]. Ectopic HIP1R overexpression led to increased invasion and migration of relatively indolent prostate cancer cells [131]. Rescue experiments demonstrated that restoring expression of HIP1R attenuated the anti-migratory effect of miR-23b/-27b in aggressive prostate cancer cells [131]. Additionally, to solidify this connection, we examined HIP1R mRNA levels in the locally invaded seminal vesicles from mice bearing orthotopic xenografts of prostate cancer cells expressing miR-23b/-27b. These studies revealed that HIP1R mRNA is significantly decreased in the seminal vesicles from mice bearing the miR-23b/-27b xenografts compared to controls. As expected, miR-23b and miR-27b levels were higher in these samples. Together these data indicate that the anti-metastatic actions of miR-23b/-27b in prostate cancer cells is due, at least in large part, to repression of HIP1R levels.

Although HIP1R is a predicted target of miR-23b/-27b, luciferase reporter assays suggested miR-23b/-27b may not directly regulate HIP1R via its 3'UTR. While 3'UTR binding is the canonical mechanism of miRNA target binding, it is possible that the essential regulatory sequences of HIP1R lie outside of this region [147]. This less common localization of miRNA targeting sequences has
been documented for other mRNAs regulated by miRs, such as the regulation of Nanog by miR-296 [148]. However, we were able to express a recombinant version of HIP1R that lacks the 3' UTR in the presence of miR-23b/-27b suggesting that there are no miR-23b/-27b binding sites in the coding sequence of the gene. Additionally, our collaborator Dr. Derek Dykxhoorn performed thorough analysis of miR-23b and miR-27b binding sites in the HIP1R gene using the IntaRNA (Interacting RNA) program [149], which assesses the binding of the query RNA (in this case the miRNAs) and a user defined target (in this case the genomic sequence of the longest isoform of the HIP1R gene including 5' UTR, all exons, all introns, and the 3' UTR). This program identified 5 putative binding sites for both miR-23b and miR-27b that had predicted binding interactions with strong seed matches, all of which mapped to intronic sequences of HIP1R. This was not surprising as the introns in the HIP1R gene make up over 80% of the gene. However, it is unlikely that these binding interactions would take place since the introns are spliced out of the primary transcript of the gene and only the mature RNA lacking introns is exported to the cytoplasm, where miRs associate with RISC. Although miRs can be expressed and processed from intronic sequences, there is no evidence that miRNAs can target intronic sequences in mammals.

Cycloheximide assays were performed to confirm miR-23b/-27b regulate HIP1R indirectly, with an intermediary gene, by determining if new protein synthesis is required for miR-23b/-27b mediated HIP1R mRNA regulation. I carried out these assays testing a variety of experimental paradigms but had
issues with consistency—likely due to complications related to the effects of
cycloheximide on the miRNA processing proteins themselves. Expression of the
miRs depends on Drosha, as well as incorporation into RISC, which itself
involves interaction with Argonaute, therefore, any alteration of new protein
synthesis will affect these processes, preventing proper processing of mature
miR-23b/-27b. The successful assays in which HIP1R mRNA was down-
regulated by miR-23b/-27b in untreated samples utilized transduction of mature
miR-23b and miR-27b compared to our previous models of incorporating the
unprocessed miR-23b/-27b precursor. This was with the goal in mind of
circumventing the majority of miR processing pathways and limiting unintended
effects of cycloheximide. This experiment demonstrated miR-23b/-27b repressed
HIP1R mRNA, which was attenuated upon treatment with cycloheximide. This, in
addition to the 3’UTR transcriptional output assay results strongly suggest that
miR-23b/-27b do not directly target HIP1R mRNA.

Instead, it is possible that miR-23b/-27b target an as of yet unidentified
gene, which lies upstream of HIP1R. For example, if miR-23b/-27b directed the
silencing of a transcription factor that was required for the transcription of HIP1R,
the silencing of this transcription factor would result in a concomitant decrease in
HIP1R expression at both the mRNA and protein levels. This spurred us to
investigate known transcription factors for HIP1R, as we observed [132]. One
such protein, FOXP1, a member of the FOXP family commonly associated with
immune response [150] is known to repress HIP1R in Diffuse Large B-Cell
Lymphoma (DLBCL) [151]. FOXP1, is also a known tumor suppressive gene in
prostate cancer tumors, negatively regulating the androgen receptor (AR) [152, 153]. Our aforementioned microarray indicated miR-23b/-27b overexpression led to increased FOXP1 levels in aggressive prostate cancer cells, but this link has yet to be confirmed in vitro. If upon further exploration miR-23b/-27b regulates FOXP1 in prostate cancer cells it would provide a possible mechanism through which HIP1R acts on metastasis suppression. It is important to note that FOXP1 is upregulated in the presence of miR-23b/-27b in PC3-ML cells. This would suggest that miR-23b/-27b does not directly regulate FOXP1, as miRs transcriptionally repress a direct downstream target gene. Fittingly, there are no predicted binding sites of miR-23b, miR-27b or miR-24 on FOXP1. Thus, introducing an additional mediator in the mechanism of HIP1R regulation. Nevertheless, our data support HIP1R down regulation as a key component of miR-23b/-27b anti-metastatic actions.

Another intermediary gene candidate is Homeodomain-interacting protein kinase 3 (HIPK3), a serine/threonine kinase involved in transcriptional regulation, apoptosis and steroidogenic gene expression. Using DIANA Tools, based upon TarBase v7.0, which identifies proven miRNA targets by indexing known miRNA:mRNA interactions [154], I established that HIPK3 is a known target of miR-27b [155-157], as well as miR-24 [158]. Upon further investigation, HIPK3 is increased by JNK activity in prostate cancer cells (DU145), contributing to FAS receptor-mediated apoptosis [159]. HIPK3 is also linked to multi-drug resistance in several tumor types [159], and our microarray studies found HIPK3 to have a 1.3 fold decrease (P=0.021) in PC3-ML cells overexpressing miR-23b/-27b.
Interestingly, HIPK3 is a target of FOXO1, of which FOXP1 can directly modulate [160].

The mechanisms by which HIP1R promoted invasion, migration and anoikis-resistance are currently unknown. However, the demonstrated role of HIP1R as an adaptor of clathrin-mediated endocytosis provides several novel possibilities. HIP1R may increase migratory and invasive properties of prostate cancer cells by promoting internalization of cell surface molecules such as E-cadherin, dynamin, and/or integrins at sites of cell-cell contact, adherens junction or focal adhesions; regulating recycling of membrane proteins, or modulating the internalization, trafficking, stability and/or signaling of growth factor/cytokine receptors. Constitutive endocytosis of death receptors has been implicated in breast cancer cells which undergo resistance to TRAIL receptor-mediated therapies, implicating clathrin dependent endocytosis as a possible therapeutic target for tumor resistance [161]. As deregulation of endocytosis is increasingly recognized as an important driver of cancer progression [117], future studies should address whether HIP1R involvement in this key pathway leads to metastasis.

Clathrin dependent endocytosis (CDE) is the best characterized endocytic pathway, as macromolecules such as lipoprotein-, transferrin- and epidermal growth factor receptors preferentially associate with this pathway and are taken up in clathrin-coated vesicles [118]. Therefore there are many viable approaches
of testing HIP1R’s functionality in endocytosis. Common inhibitors of CDE include chlorpromazine and potassium depletion. Chlorpromazine inhibits clathrin-coated pit formation by a reversible translocation of clathrin and its adapter proteins from the plasma membrane to intracellular vesicles [162], while potassium depletion dissociates the clathrin lattices at the inner leaflet of the plasma membrane [163]. Methyl-β-cyclodextrin (MβCD) and genistein are evaluated as inhibitors of clathrin-independent endocytosis [164]. MβCD is regularly used to determine whether endocytosis is dependent on the integrity of lipid rafts. Genistein is a tyrosine-kinase inhibitor and this agent causes local disruption of the actin network at the site of endocytosis and inhibits the recruitment of dynamin II [165, 166]. Additionally, Dynamin is an intracellular GTPase essential for membrane remodeling, fission of clathrin coated vesicles in endocytosis, as well as vesicles budding from the trans-golgi network [167-169]. Dynasore, a dynamin inhibitor, targets the GTPase activity of Dynamin 1, inhibiting endocytosis of the transferrin receptor and LDLR, as well as suppresses cancer cell invasion through destabilization of F-actin by inhibiting formation of pseudopodia [169, 170]. Some limitations of Dynasore included toxicity due to binding of serum proteins, as well as reduced potency in in vitro drug screens [168], prompting development of less toxic analogs called Dyngo [171]. Alternatively, HIP1R’s function as pertaining to invasion and migration could easily be explored with the use of functional domain mutants. The lab of David Drubin has developed multiple domain mutants of HIP1R [172]. Migration assays could be performed overexpressing HIP1R functional mutants to the
ANTH domain- involved in clathrin association, coiled-coil domain- responsible for dimerization and clathrin-light chain binding, as well as the thatch domain, where actin binding occurs, in the LNCaP cell line.

Our lab previously demonstrated miR-23b/-27b regulation in prostate cancer cells alters E-Cadherin protein and RNA levels [97, 131]. Unexpectedly, this phenotype was not recapitulated for HIP1R in my studies. This may suggest that HIP1R may not be responsible for the entirety of miR-23b/-27b’s metastasis suppressing phenotype. E-cadherin is removed from the plasma membrane by endocytosis and recycled to sites of new cell-cell contacts [20]. Given HIP1R’s known involvement in endocytosis, it stood to reason that regulation of E-Cadherin could be occurring via alterations in subcellular localization. I posited that loss of HIP1R would prevent endocytic turnover of E-Cadherin, and result in accumulated cell surface E-Cadherin. Preliminary experiments suggest that qualitatively, this may not be the case, although additional experiments assaying endocytosis, such as transferrin uptake assays would provide a more in-depth look at regulation of cell surface proteins.

Abnormal activation of proto-oncogenes, such as c-Met, Src, and EGFR, results in increased phosphorylation of tyrosine residues in the cytoplasmic domain of E-cadherin, which leads to recruitment of the E3-ubiquitin ligase Hakai and subsequently mediates internalization and ubiquitin-dependent degradation of E-cadherin [173]. Proteolytic degradation of E-cadherin by matrix metalloproteases (MMPs) is another mechanism by which E-cadherin-mediated cell–cell adhesion can be ablated. A soluble 80-kDa form of E-cadherin,
produced by the degradation of the full-length protein (degradation products observed in Figures 21 and 22), is frequently found in cultured tumor cell lines and in tumor biopsy samples. This soluble form of E-cadherin promotes tumor-cell progression and invasion by upregulating MMPs, such as MMP2 (required for Rac1 promoted cell invasion through collagen barriers [174]), as well as MMP9 and MMP14 [175]. Despite the fact that miR-23b/-27b regulate E-Cadherin, it occurs independently of Matrix Metalloproteases [131] and HIP1R level manipulation leading to expression of the 80kDa E-Cadherin fragment, HIP1R’s effects on metastatic properties appear to be independent of E-Cadherin. Interestingly, miR23a directly binds to 3’UTR of E-cadherin inducing EMT, although there are conflicting reports of miR-23a levels in prostate cancer [94, 176].

It is of note that the phenotype of HIP1R knockdown differs between PC3-ML and ALVA31 cells. ALVA31 are a more mesenchymal cell line that is fast growing, but not commonly associated with metastasis, while PC3-ML are the metastatic derivative of an already aggressive cell line, PC3. These cells are slower growing, but have high metastatic potential. Upon HIP1R knockdown, ALVA31 exhibited decreased Rac1 activity, as well as a significant shift in cell morphology, that was not observed in PC3-ML. Rac1 is known to regulate adherens junctions through endocytosis of E-Cadherin [177], and there is precedent for Rac1 to be regulated by miRs, such as by miR-320a in colorectal cancer [178]. Additionally, pathway analysis of our microarray studies using GenPath UM pathway analysis (bio.ccs.miami.edu), determined that our gene list
was significantly enhanced for involvement in adherens junctions (enrichment 8.47, P=0.005).

Out of interest in determining the direct target of miR-23b/-27b responsible for the anti-metastatic phenotype in prostate cancer, we collaborated with Drs. Yassin Flores and Brian Cullen at Duke University to perform Phosphoactivatable Ribonucleoside Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) analyses [179]. This technique isolates mRNA sequences bound to RISC (pulling down for Argonaute) at the time of cross-linking, allowing for transcriptome-wide identification of miR target sites. These sequences are then compared to known seed sequences for miRNA of interest, and scanned for the number of reads. Genes with high read counts are then said to be predicted direct targets of these miRs. For our studies, Reema Ishteiwy prepared PC3-ML cells overexpressing miR-23b/-27b and compared them to LNCaP cells, which have high endogenous miR-23b/-27b expression. I then compared the experimental output by determining gene targets that overlap in the miR-23b/-27b positive PC3-ML cells and LNCaP, yet there were no common genes between the PAR-CLIP and microarray studies. This could occur for a number of reasons- including high stringency of our assay output, or lack of a PC3-ML control sample. A generally applicable method for data analysis of CLIP experiments is estimating the enrichment of reads in relation to relative mRNA abundance [180]. Doing so, I was able to ascertain an idea of pathway regulation attributed to these predicted targets using GenPath UM pathway analysis (bio.ccs.miami.edu), as the gene list was significantly enriched for involvement in Glycerophospholipid metabolism
(P=0.0004), Actin cytoskeleton (P=0.0026), and to a lesser degree, sphingolipid, glycerolipid and purine metabolism (P<0.05).

Interestingly, of the 25 genes overlapping between PC3-ML miR-23b/-27b and LNCaP (Table 2), 23 were predicted targets of miR-27b, while only 2 were predicted targets of miR-23b. While we know the effects of the miR-23b/-27b cluster to differ from individual miR-23b or miR-27b expression, this disproportionate targeting in favor of miR-27b may suggest that the bulk of the phenotypic effects on prostate cancer cells may fall to miR-27b. Furthermore, miR-27b expression and not miR-23b was a good disease progression marker in prostate cancer [181]. Individual effects of miR-27b should be further examined in prostate cancer, by transfecting miRNA mimics to miR-27b, followed by invasion and migration experiments, compared to miR-23b alone, or miR-23b/-27b in combination. If the anti-metastatic phenotype is supported by miR-27b alone, it may stand to reason to follow up on a single miR for therapeutic targeting to limit unintended off-target effects.

**MiRNA Therapeutic Potential and Delivery**

We have demonstrated the potential of miR-23b/-27b as a preventative treatment for metastatic prostate cancer, yet the question remains of how to translate these finding to patient care. Treatment of diseases with small RNA’s, and miRNAs in particular as therapeutic targets is a rapidly expanding area of study, but delivery methods for these small RNAs are still in their infancy. The first hurdle of delivering therapeutic siRNAs was reaching the tissue of interest,
which required overcoming several physiological barriers such as renal clearance, degradation in serum and cell-specific uptake, as well as intracellular barriers in the form of saturating endogenous RNA processing machinery, and endosomal escape, reducing efficiency of the siRNA [182]. These issues have contributed to strong immunotoxicity in early attempts. Ultimately, delivery vehicles including liposomes and lipid-based nanoparticles (LNPs) have helped siRNA lower toxicity as well as produce a more stable therapeutic with lower production costs, making the marketability of siRNA therapy a reality. The first lipid-encapsulated chemotherapeutic drug, Doxil, was approved in 1995, yet very few have since past FDA inspection due to lack of efficiency, toxicity issues, and chemistry/manufacturing/control [183].

Lipids form the core components of nanocarriers for siRNA delivery, allowing scientists to take advantage of “passive targeting” from neovascularization and poor lymphatic drainage common in tumor physiology. Additional benefits to nanocarriers include the amount of drug administered per liposome (ex: 10,000 doxorubicin molecules per one liposome or 4,000 siRNA mol per lipid based nanoparticle [184]), protection from the internal microenvironment, as well as timed-release of the drug as a means to decrease patient dosing and increase quality of life. One of the main problems that remain is how to actively target these nanoparticles to cancer cells, allowing for greater cargo delivery, specificity and minimization of off-target effects. Scientists are actively taking advantage of cancer-associated antibodies, aptamers, and cell surface proteins including peptides and natural ligands. A common strategy
involves targeting hyaluronan receptors, CD44, commonly overexpressed on many tumors. Hyaluronan is beneficial as a hydrophilic coating, promoting longer circulation in addition to providing high-affinity binding to tumor localized recognition sites [185].

The first miRNA based therapy for cancer is a miRNA mimic to miR-34 (MIRX34), which is encapsulated in a proprietary liposomal based delivery system called SMARTICLES. MRX34 is currently in expansion cohorts of Phase 1 clinical trials for treatment of solid tumors including hepatocellular carcinoma, melanoma, small and non-small cell lung cancer (SCLC, NSCLC) as well as hematological malignancies, lymphoma, and multiple myeloma, with plans to initiate phase 2 trials beginning in 2017 (mirnarx.com). Nevertheless, miR-based-therapeutics are still in their infancy and the side effects of these therapies need to be carefully evaluated [186]. Looking forward, focus should remain on safety, as well as biological stability, and new tumor targeting strategies to aid in stability.

Ultimately, miRs are a complex field with specific contextually dependent outcomes on downstream processes. Not only do miRs within the same cluster or family possess varied potential, but the same miR in different diseases poses just as many possibilities. miR-23b-27b-24-1 has an evolutionarily conserved paralog, miR-23a-27a-24-2 found on chromosome 19. This “a” cluster, whose members miR-23a and -27a only differ from their miR-23b and miR-27b paralogs by one nucleotide (miR-24 is identical), has conflicting reports in prostate cancer. miR-23a has been found to upregulated in prostate cancer in some studies [176,
187, 188], and down-regulated in other studies on prostate cancer cells and tumor tissues [189, 190]. miR-23a-27a-24-2 as a whole even acts as an “oncomiR” in prostate cancer and is transcriptionally upregulated by AR unlike miR-23b-27b-24-1 [191]. Contextual dependency highlights the importance of personalizing medicine to the individual cancer, or even tumor. Identifying markers, such as low miR-23b/-27b that could preemptively identify the cases in which patients are more likely to progress to aggressive disease affords huge benefit to fields such as prostate cancer in which patients are often over-treated leading to worse prognoses. Likewise, development of new small molecules specifically targeted to tumors improves the cancer field as whole.
REFERENCES


119. Loop SM, Rozanski TA, Ostenson RC. Human primary prostate tumor cell line, ALVA-21: A new model for studying the hormonal regulation of prostate tumor cell growth. The Prostate. 1993;22(2):93-108.


131. Ishteiwy RA. MiR-23b/-27b cluster suppresses the metastatic phenotype of aggressive prostate cancer cells 2013.


